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SHORT COMMUNICATION

ADENOSINE RELEASE IN THE ANOXIC TURTLE BRAIN: A POSSIBLE MECHANISM FOR ANOXIC SURVIVAL

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In the brain of most vertebrates, anoxia or ischaemia rapidly causes a fall in ATP levels and in the activity of the Na^+/K^+ pump, resulting in general depolarization followed by a cascade of catastrophic events (Hansen, 1985; Hochachka, 1986). By contrast, the brain of freshwater turtles survives many hours of complete anoxia at 25°C. Hence, the turtle brain has become the archetype of an anoxia-tolerant brain, and considerable efforts have been devoted to finding the mechanisms underlying its exceptional ability to survive without oxygen. The turtle brain's main strategy for anoxic survival seems to be to decrease energy utilization, so that ATP consumption can be met by glycolytic ATP production alone (Sick *et al.* 1982; Lutz *et al.* 1985; Chih *et al.* 1989). However, the mechanisms mediating the lowered energy consumption have remained obscure.

Neurotransmitters are ubiquitous regulators of brain activity. We recently found that, in response to anoxia, the turtle brain increases the levels of the inhibitory neurotransmitters/neuromodulators γ-aminobutyric acid (GABA), glycine and taurine, while simultaneously reducing the level of the excitatory neurotransmitter glutamate (Nilsson *et al.* 1990). Moreover, by the use of microdialysis, we have also observed increases in the extracellular concentrations of GABA, glycine and taurine in the anoxic turtle brain (Nilsson and Lutz, 1991). However, the increase in the tissue levels of these inhibitory amino acids were relatively slow, and their release to the extracellular space was only apparent after about 100 min of anoxia. Thus, while inhibitory amino acids may be important mediators of the long-term suppression of energy use seen in anoxic turtles, they are not likely to be responsible for the initial early decrease in brain activity needed to meet a falling rate of ATP production.

Consequently, we started to look for another inhibitory factor that would show a faster response to anoxia. One such factor could be adenosine, a product of the

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breakdown of high-energy purines like ATP. Indeed, two previous studies on the brain of anoxic turtles (Lutz *et al.* 1984; Kelly and Storey, 1988) have shown that there is a small but significant temporary fall in ATP, ADP and AMP levels as an immediate response to anoxia. This suggests that there might be a corresponding increase in the level of adenosine.

Adenosine is an inhibitory neuromodulator in the vertebrate brain, decreasing neuronal excitability (postsynaptic inhibition) as well as neurotransmitter release (presynaptic inhibition) (Stone, 1981; Proctor and Dunwiddie, 1987). Moreover, adenosine is known to cause cerebral vasodilation, thereby increasing cerebral blood flow (Berne *et al.* 1974; Morii *et al.* 1987) and, more recently, it has been found to stimulate glycogenolysis (Magistretti *et al.* 1986). Thus, adenosine seems to have all the properties required for promoting anoxic survival. Interestingly enough, the anoxic turtle brain displays a decrease in electrical activity (Sick *et al.* 1982) and synaptic transmission (Feng *et al.* 1988), as well as an initial 10-fold increase in cerebral blood flow (Bentley, 1986). Nevertheless, a possible role for adenosine in the anoxia tolerance of the turtle brain has not been examined previously.

In the present study, we have measured the effect of anoxia on the extracellular level of adenosine in the turtle brain striatum. Extracellular fluid was sampled by microdialysis *in situ* (Ungerstedt, 1984).

Turtles (*Pseudemys scripta* Schoepff, weighing 350–500 g) were anaesthetized (pentobarbital 50 mg kg^{-1} intraperitoneally) and ventilated with air (using a small-animal respirator from Phipps and Bird Inc., Richmond, VA) while a 5 mm×5 mm area of the skull above the telencephalon was removed. A small hole was made in the dura and, using a stereotaxic instrument, a microdialysis probe (1 mm membrane length, from Carnegie Medicin, Stockholm, Sweden) was gently pushed down through the roof of one of the telencephalic hemispheres to the striatum (2.0 mm from the midline of the brain, 1.5 mm behind the rostral tip of the telencephalon and 4.0 mm ventral to the dura). The probe was perfused at a rate of 1 $\mu l\,min^{-1}$ using a CMA-100 microinfusion pump (Carnegie Medicin, Stockholm, Sweden). The perfusion Ringer contained 108 mmol l^{-1} NaCl, 26 mmol l^{-1} $NaHCO_3$, 3.5 mmol l^{-1} KCl, 1.7 mmol l^{-1} NaH_2PO_4, 1.5 mmol l^{-1} $CaCl_2$ and 0.7 mmol l^{-1} $MgSO_4$ (pH 7.4). Anoxia was produced by feeding N_2 into the respirator. After each experiment, the probe was perfused with Methylene Blue and the brain was removed, frozen and sliced to confirm the position of the probe. The dialysis fluid was collected at 30 min intervals and analysed with regard to the content of adenosine using HPLC with spectrophotometric detection, as described by Hagberg *et al.* (1987). The values were adjusted for the *in vitro* recovery (10–14 %) measured for each probe, as described by Tossman and Ungerstedt (1986).

Using the same experimental set-up, the extracellular concentration of alanine, an end product of anaerobic metabolism, was measured to obtain a marker for the onset of anoxia in brain tissue under the present conditions (Nilsson and Lutz, 1991).

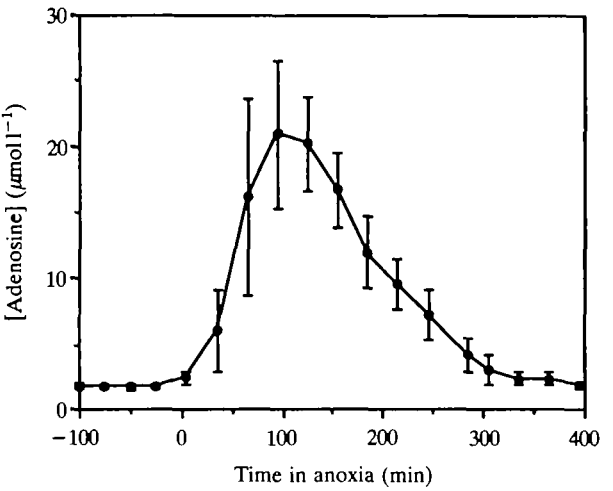

Fig. 1. Effect of N_2 respiration on the extracellular level of adenosine measured by intracerebral microdialysis in the striatum of freshwater turtles held at 25 °C. The onset of anoxia (time=0 min) was counted from the extrapolated start of the rise in the concentration of alanine (occurring after 40 min of N_2 respiration), an end product of anaerobic metabolism (see Fig. 2). Values are mean±S.E.M. (N=4).

The effect of N_2 respiration on the extracellular level of adenosine in the striatum of freshwater turtles is shown in Fig. 1. The normoxic level of adenosine was 1.7±0.2 μmol l^{-1}. Immediately upon the onset of tissue anoxia, the extracellular level of adenosine started to increase, peaking at 20.9±5.7 μmol l^{-1} after 90 min (12 times the control level). The adenosine concentration then fell, returning to the control level after 240–400 min of anoxia. In the brain of turtles respirated with air for 150 min, the extracellular concentration of adenosine did not increase.

The onset of tissue anoxia occurred 40±13 min (N=4) after the start of N_2 respiration, as calculated from the extrapolated start of the alanine rise (Fig. 2). To get an accurate picture of the time course of the events initiated by anoxia under the experimental protocol used, a marker for the start of anoxia at the tissue level is needed (Lutz *et al.* 1984; Nilsson and Lutz, 1991). The onset of tissue anoxia is likely to vary between the different protocols used in experiments with anoxia in turtles (N_2 respiration *via* a respirator, voluntary N_2 respiration in a N_2 atmosphere, and forced submergence in anoxic water).

Adenosine release to the extracellular space in response to hypoxia and ischaemia is well established in the mammalian brain (Berne *et al.* 1974; Van Wylen *et al.* 1986; Hagberg *et al.* 1987), an event that is thought to be a direct result of the massive breakdown of phosphorylated purines (ATP, ADP and AMP) during oxygen depletion (Barberis *et al.* 1985; Hagberg *et al.* 1986; Newby *et al.* 1990). Hence, much interest has lately been focused on a possible role of adenosine in counteracting hypoxic or ischaemic brain damage in mammals (Newby *et al.* 1990), although the ability of adenosine to do this in mammals must

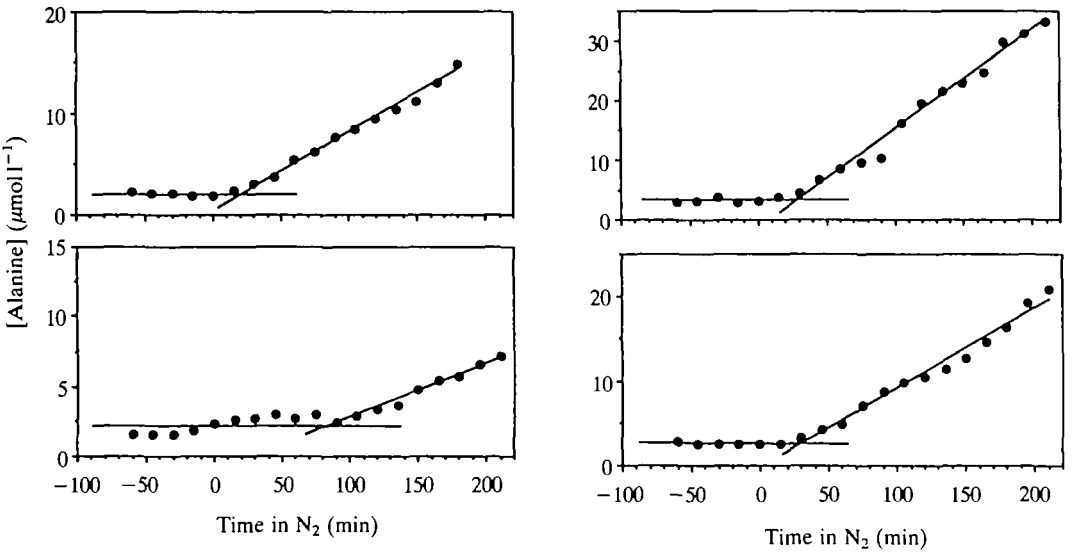

Fig. 2. Effect of N_2 respiration on the extracellular level of alanine measured by intracerebral microdialysis in the striatum of four freshwater turtles held at 25°C. The extrapolated start of the rise in alanine concentration suggested that the onset of anoxia at the brain tissue level occurred after 40±13 min of N_2 respiration (mean ±S.E.M.).

be limited, as evident from the high susceptibility of the mammalian brain to anoxic damage. Unlike adenosine release in the mammalian brain, the release in turtle brain may truly be a mechanism for anoxic *survival*.

In contrast to the almost total depletion of phosphorylated purines in the anoxic mammalian brain, the turtle brain maintains its levels of ATP, ADP and AMP relatively constant throughout many hours of anoxia. Nevertheless, two previous studies have shown that there is a limited, but significant, temporary fall in the levels of these energy-carrying compounds. Hence, Lutz *et al.* (1984) found that the turtle brain levels of ATP and ADP decrease by 19% and 38%, respectively, after 10 min of N_2 respiration, while normal levels were re-established after an additional 35 min of N_2 respiration. Likewise, Kelly and Storey (1988) observed 26%, 35% and 78% falls in ATP, ADP and AMP concentrations, respectively, after 1 h of submergence in anoxic water, while 5 h of submergence caused the ATP and ADP levels to increase above the normoxic levels. This temporary breakdown of phosphorylated purines is likely to be the most important source of the increase in extracellular adenosine concentration reported in this paper. A comparison with the previous data suggests that there is a delay between the fall in intracellular phosphorylated purines and the elevation in extracellular adenosine concentration in turtle brain. A similar, but shorter, delay is also seen in the mammalian brain (Hagberg *et al.* 1987).

As mentioned above, adenosine seems to be an ideal candidate for a mediator of anoxic survival in the turtle brain, because of its ability to decrease electrical and

synaptic activity, elevate glucose levels and increase cerebral blood flow. Moreover, adenosine induces bradycardia in rats, when injected in brain areas responsible for the regulation of heart rate (Tseng *et al.* 1988). Indeed, turtles display a large fall in heart rate after about 1 h of submergence in deoxygenated water (Penney, 1974).

Although both the mammalian and the turtle brain respond to anoxia by releasing adenosine, only turtles have the ability to avoid a general depolarization and survive prolonged anoxia. Clearly, a major factor underlying this discrepancy is the exceptional ability of turtles to maintain the brain energy charge by anaerobic glycolysis alone. Experiments with brain slices at 26 °C indicate that the normoxic turtle brain depends more on glycolytic ATP generation (24 % of total ATP production) than does the normoxic rat brain (13 % of total ATP production) (Robin *et al.* 1979). Thus, the turtle brain has to make a smaller increase in glycolytic rate to compensate for the cessation of aerobic energy production. Moreover, at their respective physiological body temperatures (37 °C and 21 °C), the rate of normoxic oxygen consumption by mammalian brain synaptosomes is about four times higher than that of turtle brain synaptosomes (Edwards *et al.* 1989). Consequently, in the anoxic turtle brain, the effects of adenosine (reducing energy consumption and increasing energy supply) combined with a high basal glycolytic activity, as well as glycolytic activation (Kelly and Storey, 1988), could be enough to compensate for the loss of aerobic energy production.

The response of the turtle brain to anoxia appears to occur in two phases (Lutz *et al.* 1985). During the initial transition phase, the loss of oxidative phosphorylation is compensated by enhanced glycolysis (Kelly and Storey, 1988). This phase is followed (after approximately 1 h) by a radical down regulation where energy requirements are reduced to about 5 % of normoxic values (Chih *et al.* 1989).

The increase in extracellular adenosine in the turtle brain was only temporary, and the adenosine level started to fall after 90–120 min (Fig. 1). We have recently found that the extracellular levels of the inhibitory amino acids GABA, glycine and taurine start to increase after about 100 min of anoxia (Nilsson and Lutz, 1991), i.e. at about the same time as the adenosine level starts to fall. Thus, it is tempting to suggest that adenosine mediates the initial compensatory energetic shift in turtle brain by reducing brain activity and increasing glucose supply, while the later release of inhibitory neurotransmitters plays a role in establishing and maintaining the radically depressed metabolic state that allows the turtle brain to survive prolonged anoxia.

In conclusion, by combining the present results with those of previous studies, a model for the anoxic survival of the turtle brain emerges, where the following events appear to be the most fundamental. By blocking oxidative energy production, anoxia causes the rate of ATP synthesis to fall below the rate of ATP use. As a result, adenosine will be formed as phosphorylated purines like ATP are degraded. Adenosine will increase the rate of glycogenolysis and brain blood flow, hence increasing the amount of glucose available for glycolytic ATP synthesis. Simultaneously, adenosine will cause a decrease in ATP use by inhibiting neuronal

excitability and transmitter release. Consequently, the ATP level will rise again while the adenosine concentration will stabilize or fall. Finally, adenosine will return to pre-anoxic levels when brain activity has been suppressed by the increased release of inhibitory amino acids seen at a later stage of anoxia.

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